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EFFICACY OF DIFFERENT FEEDER FREE CULTURE CONDITIONS FOR DERIVATION OF GOAT EMBRYONIC CELL CLONES

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ABSTRACT

The presence of feeder cells and feeder-conditioned media of animal origin are among the major obstacles impeding successful derivation and continuous culture of embryonic cells. An attempt was made to derive goat (Capra hircus) embryonic cells in feeder freeculture conditions using gelatin, matrigel and polylisine coated plate.Four different media viz. medium-1, medium-2, medium-3 and medium-4 were used in each condition. Medium-1 was considered as standard medium containing CR11aaa supplemented with BSA (3 mg/ml), 20% FBS, LIF (40ng/ml), ITS (0.1%), IGF-1 (100ng/ml), bFGF (4ng/ml), EAA (1%) and NEAA (0.5%). Other media were prepared by slight modification of medium 1. Medium-2 was prepared by reducing FBS content to 10%; Medium-3, NEAA content was increased to 1% by withdrawing EAA and medium-4 was prepared by additional supplement of glucose 0.1% (1mg/ml). A total of 32 embryos in each medium and factor coated conditions were cultured in 8 different well comprising four embryos in each well. Mean (±SE) embryos per well developed to cell clones were 1.625±0.5536, 1.375±0.2679, 1.875±.4107 and 1.375±0.2679 in medium 1, 2, 3 and 4 respectively while cultured on gelatine coated plates. The corresponding value for matrigel coated plates were 1.875±0.4107, 1.250±0.2143, 1.875±0.4107 and 1.500±0.2857, respectively. No embryo was found to develop to cell clones cultured on polylysine coated plates. The developed cell clones showed positive for pluripotent specific markers of alkaline phosphatase activity, SSEA-1, SSEA-3, TRA-1-81(TRA-1-80), Oct-4 and Nanog. The overall embryos developed to cell clones were almost similar in gelatin and matrigel coated plate culture conditions in each of the medium used and ranges between 31.25 to 46.875 percent.Media containing 20% FBS (Mediun 1 & 3) were found to be slight better than medium-2 containing 10% FBS. Though statistically not, but numerically medium-3 was found to be superior followed by medium-1.

KEYWORDS: Gelatin, Matrigel, Polylysine, Blastomere, Feeder Free, Cell Clones

INTRODUCTION

The presence of feeder cells and feeder-conditioned media of animal origin are among the major obstacles impeding successful derivation and continuous culture of embryonic cells, especially human embryonic stem cell (hESC) for therapeutic purposes. The risk of contamination with xenopathogens makes these stem cells unsafe for further use in regenerative medicine. In present days, propagation of hESC has become possible using mammalian or human-derived extracellular matrix (ECM) like matrigel, polylysine and biomolecule like gelatin. Matrigel is a gelatinous protein mixture secreted by mouse tumor cell, which is being used extensively throughout the world (Xu et al., 2001; Ilic, 2006; Hakala et al., 2009)for coating of cell culture plates. Matrigel proteins self-assemble producing a thin film that covers the surface of the labware and give adhesive properties on the cells cultured on it. Xu et al. (2001) first describe feeder cell free hESC

culture medium conditioned with matrigel and many workers have cultured successfully ES cells on matrigel coated plate (Daheron et al., 2004; Stojkovic et al., 2004; Hysolop et al., 2005; Ilic, 2006; Zhang et al., 2009).Gelatin coating is used to coat the growth surface and commercially available as polystyrene Petri dishes and proposed for successful cultivation of human skin fibroblasts (Nizheradze and Evdokimova, 1989). The embryonic stem cells remained undifferentiated after culture on the gelatin/copolymer-coated surfaces, similar to standard culture techniques (Loh et al., 2009). William et al. (1988) developed mouse ES cells directly on gelatin coated plates with addition of leukemia inhibitory factor (LIF). Polylysine (ε-poly-L-lysine) is a small polypeptide of the essential amino acid L-lysine that is produced by bacterial fermentation. Polylysine is commonly used to coat tissue cultureware as an attachment factor which improves cell adherences.Feeder free culture is highly important to get contaminated free embryonic stem cells to be used in various research and specially for therapeutic purposes. Most of the researches in this field are limited to human embryonic cells and laboratory animals; less has been reported in domestic animals.Hence, an attempt was made to derive and culture goat embryonic cells in three different feeder free conditions of gelating, matrigel and polylysine coated plates using different media.

MATERIALS AND METHODS

Goat in vitro embryos were produced by adopting in vitro maturation, fertilization and culture (IVMFC) of presumptive zygotes. The formed zygotes were allowed to develop up to 8-16 cells stage and then processed for isolation of blastomeres. Blastomeres were isolated by treating the embryos (8-16 cells) with proteinase-K(0.2 mg/ml) made in PBS and observed for dissolution of zona pellucida under stereo zoom microscope. After 8-10 min, zona dissolution was completed and the proteinase-K activity was neutralized by adding FBS and repeated washing with TCM 199 containing 20% FBS. Finally, the blastomere clumps were washed in drop of CR11aa with 20% FBS and cultured separately in three types of feeder free conditions of gelatin, matrigel and polylysine coated plates.

The feeder free coated plates were prepared in the laboratory as described below-

- Gelatin coated plate: Gelatin (0.1%) was filled in the wells so that it covers the surface sufficiently. The plates were then kept overnight in the CO_2 incubator. Before use, the wells were washed with the medium for 3-4 times and isolated cells were cultured.
- Polylysine coated plate: The wells of the plates were filled with polylysinecovering the surface sufficiently and allowed to dry in the incubator for overnight. The wells were washed with medium for 3-4 times before seeding of the cells.
- Matrigel coated plate: The culture plates were filled with 5% Matrigel prepared in DMEM so that it covers the surface sufficiently. The plates were kept overnight at 4°C. The plates were allowed to warm to room temperature in the BOD incubator for approx 30min prior to use.

In each culture condition, four different media had been used viz. medium-1, medium-2, medium-3 and medium-4 (Table I). Medium-1 was considered as standard medium containing CR11aaa supplemented with BSA (3 mg/ml), 20% FBS, LIF (40ng/ml), ITS (0.1%), IGF-1 (100ng/ml), bFGF (4ng/ml), EAA (1%) and NEAA (0.5%). Other media were prepared by slight modification of medium 1. Medium-2 was prepared by reducing FBS content to 10%; Medium-3, EAA was totally replaced by increasing supplementation of NEAA to 1% and medium-4 was prepared by additional supplement of glucose 0.1% (1mg/ml).A total of 32 embryos were used in each media of a particular coated factor with eight (8)

replication containing four embryos in each culture well. The blastomeres were cultured under CO_2 incubator at 5% CO_2, 21% O_2, 38.5°C and 95% relative humidity. Observations and media replacement was carried out at every 48 hrs under microscope to study development of cell clones from cultured blastomeres. The developed cell clones were passaged at every six days and maintained up to second passages (18-20 days). The cell clones were characterized for pluripotent stem cell specific markers of alkaline phosphatase activity (AP) and immunocytochemistry for SSEA-1, SSEA-3, TRA 1-81 (TRA-1-80), Oct-4 and Nanog and also expression of Nanog and Oct-4 along with β-actin was assessed using PCR technique.

Synthesis of cDNA for Oct 4 and Nanog (Cell to cDNA kit, Invitrogen, USA)

Cell at 6 days of primary culture (P_0), 1st passage (P_1) and 2nd passage (P_2) were pooled. The cell clones were thoroughly washed in cold PBS for about 7-8 times and put on PCR tube. Spinning was done for about 5mins in spinner and supernatant was discarded. To this, 1μl of lysis enhancer/μl of sample and 10μl of resuspension buffer/μl of sample were added. The sample was incubated at 75^{o}C for 10 min and then spun. The sample was added with 5μl of DNase I and 1.6μl of DNase I buffer followed by incubation at 25^{o}C for 5 min and then spun for 5 minutes. Again 4μl of EDTA was added to this product and incubated at 70^{o}C for 10 min. Later, 20μl RT reaction mix and 2μl of enzyme mix was added, spun for 5 min and incubated at 25^{o}C for 10 min, 50^{o}C for 20 min and 85^{o}C for 5 min. Finally, 1μl of RNase H was added and incubated at 37^{o}C for 20 min. After incubation the sample (cDNA) was chilled and kept at -20^{o}C for further use.

Standardization of PCR

PCR was done for the identification of gene using primers for β-actin (369bp) Oct-4 (398bp) and Nanog (211bp) (Table II). Reaction mixture components, the primer concentration (10pmol/μl) and the thermal programming were standardized by putting repeated reactions so that PCR product contained only the sharp amplification of specific size without any traces of primer dimer. The standardized reaction mixture contained cDNA as target (275ng), forward and reverse primers (1.0μl each from working stock, 10pmol/μl), 12.5μl and master mix 12.5μl and rest nuclease free water to make the final volume 25μl in the PCR tube. The thermal conditions were as follows, initial denaturation at95°C for 5min followed by 40 cycles consisting denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec and extension at 72° C for 1 min, final extension at 75° C for 5 min.

The PCR product was visualized by running on 2% agarose gel with ethidium bromide (EtBr) using 50 bp plus ladder.

RESULTS

The mean (±SE) embryos per well developed to cell clones are presented in the graph (Figure 1) and were found to be 1.625±0.5536, 1.375±0.2679, 1.875±.4107 and 1.375±0.2679 in medium 1, 2, 3 and 4 respectively while cultured on gelatine coated plates. The corresponding value for matrigel coated plates were 1.875±0.4107, 1.250±0.2143, 1.875±0.4107 and 1.500±0.2857, respectively. No embryo was found to develop to cell clones while cultured on polylysine coated plates. Overall performance of development of embryonic cell clones (Table III) cultured on gelatin coated plates were 40.625%, 34.375%, 46.875% and 34.375%in medium 1, 2, 3 and 4, respectively. The performances on matrigel coated plates were 46.875%, 31.25%, 46.875% and 37.50% in medium 1, 2, 3 and 4, respectively. Statisticallyperformances were almost similar between media as well as culture conditions. However, medium-1 and medium-3 containing 20% FBS were found to be numerically slight better than other media. The cell clones developed

were passaged at every six days and found to develop to further clones up to second passages (18-20 days) in respective feeder free culture conditions.The cell clones on matrigel, gelatin and polylysin coated plates at different stage are shown in Figure 2,3 and 4, respectively. The passaged cells also showed potentiality to form cell clones in the subsequent passages. Irrespective of medium, cells at 6 days of primary culture (P_0), 1^{st} passage (P_1) and 2^{nd} passage (P_2) were characterized for pluripotency markers like alkaline phosphatise activity, SSEA-1, SSEA-3, TRA-1-81(TRA-1-80), Oct-4 and Nanog. Immunocytochemistry of the cell clones showed positive for alkaline phosphatase activity, SSEA-1, SSEA-3, TRA-1-81(TRA-1-80), Oct 4 and Nanog (Figure 5). cDNA of isolated cells for Oct 4 and Nanog were highly transcripted in PCR and the products showed sharp bends while visualized running 2% agarose gel using 50 bp ladder (Figure 6).

DISCUSSIONS

Amino acids play a multitude of rolein early embryonic development and have been demonstrated to facilitate improve development of in vivo or in vitro fertilized and parthenogenetic embryos in several species. Incorporation of only non essential amino acid(NEAA) of amino acids group may be sufficient for successful culture of pluripotent stem cells (Thompson et al., 1996; Amit et al., 2003). Nonessential amino acids improved the yield and quality of cloned and parthenogenetic porcine embryos and modulate the expression of embryo survival related genes. Deprivation of a set of nonessential amino acids (NEAA) potently inhibited cell cycle progression and selectively down-regulated the expression of proliferation-control proteins (Nelsen et al., 2003). Incorporation of higher concentration (1%) of NEAA to medium-3 might have attributed towards slightbetter performance in forming embryonic cell clones than other media used.Gupta et al. (2008) reported that supplementation of NEAA to culture medium improves the yield and quality of cloned porcine embryos by enhancing blastocyst expansion, hatching, and total cell number and decreasing the apoptosis by positively modulating the expression of embryo survival related genes. FBS is a common ingredient and have been used in most of the stem cell isolation and propagation at a range of 15-20%(Mitalipova et al., 2001). We tried to reduce the FBS supplementation to 10% through medium-2, but found slight poor performance as compare to medium-1 and medium-3 (FBS 20%). It has been reported that use of less FBS (10%) could result significantly ($P<0.05$) lower performance than high FBS (20%) forisolation and expansion of stem cells derived from amniotic fluids (Liu et al., 2009). Serum reduction led to a rapid decrease in the number of dividing cells (Kues et al., 2000), whereas, serum deprivation may cause apoptosis−like cell death of cultured cells (Koyama et al., 2000).Glucose supplementation to embryos and blastomere culture could give varied results (Kwun et al.,2003). Glucose is a reducing sugar that participates in Schiff base formation and Amadori rearrangements that cause protein glycation and carbonyl stress in cell culture. Weil et al. (2009) hypothesized that human mesenchymal stem cells (hMSCs) cultured in high glucose-containing media would exhibit diminished proliferation and attenuated production. Therefore, this glucose supplementation might have produced stress on these cells and could lead to low performances in forming embryonic cell clones.

Matrigel has been known as superior matrix for hESC culture over the other purified human ECM proteins, serum matrices, and the biomaterials (Hakala et al., 2009). In the present study,matrigel performances werenot found to be much better than gelatin in culturing goat embryonic blastomere cells to develop cells clones.Gelatin coating is used to coat the growth surface and is successful in cultivation of human skin fibroblasts(Nizheradze and Evdokimova, 1989). The embryonic stem cells remained undifferentiated after culture on the gelatin/copolymer-coated surfaces, similar to standard culture techniques (Loh et al., 2009). William et al. (1988)developed mouse ES cells directly on gelatin coated plates with the addition of leukemia inhibitory factor (LIF). On the other hand, polylysine is a small polypeptide of the essential amino

acid L-lysine,belongs to the group of cationic surfactants and used as food preservatives to prevent microbial growth. Polylysine can be absorbed electrostatically to the cell surface of bacteria and eventually leads to the abnormal distribution of the cytoplasm causing damage to the bacterial cell (Shima et al., 1984). This might have disrupted normal functions of the isolated cells and for which no blastomere could develop to cell clones on polylysine coated plate.

Pluripotency of developed cell clones were assessed by presence of pluripotent specific markers of alkaline phosphatise (AP) activity, SSEA-1, SSEA-3, TRA 1-81 (TRA 1-80), Oct 4 and Nanog. Currently the most widely tested and validated panel for identification of pluripotent stem cells include Oct 4, Nanog, AP activity, SSEA-3, SSEA-4, TRA-160 and TRA 1-81. The human ES cells that show positive for AP also co express Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60,and TRA-1-81 and loss of this AP occurs more rapidly than the lossof SSEA-3 or Oct-4 expression (O'Connoret al., 2008)**.**The goat ESC like cells also expressed pluripotent markers of AP activity, TRA 1-61, TRA 1-81, Oct 4, Nanog (Kumar et al, 2011), SSEA-1, SSEA-4 (Pratheesh et al., 2013), SSEA-3 (Yang et al., 2013). Guo et al. (2011) concluded that presence of Oct 4 and Nanog markers in glioma cells may indicate activation of ESC associated pathways to become pluripotent. In the present study, presence of AP activity was largely confined to the outer plasma membrane of stained cells. This might describe that the cells were at very early stage of cell cycle and formation. During the course of cell development, cell plasma membranes invaginate into the cell body to form cell cytoskeleton and give a uniform colouration, if stained for AP activity. Cells isolated from early stage embryos (8-16 cells stage) were found to be totipoten in nature and several success have already been established (Jonathan and Mervin, 2003; Bondioli et al., 1990; Strelchenko et al., 2004).Therefore, the isolated pluripotent cells were at very early stage of cell cycle and might be of totipotent in nature.

CONCLUSIONS

Extracellular matrix matrigel and gelatin can be effectively used for culture of blasomere cells to derive embryonic cells. This also may be attributedtowards the production of contaminated free purpose cells to be used for various animal research and production. Cell clones were found positivefor stem cell specific markers of alkaline phosphatase activity (AP), SSEA-1, SSEA-3, TRA-1-81(80), Oct-4 and Nanog.Medium-3 containing CR11aaa supplemented with BSA (3 mg/ml), 20% FBS, LIF (40ng/ml), ITS (0.1%), IGF-1 (100ng/ml), bFGF (4ng/ml) and NEAA (1%) was found to be better for development of embryonic cell clones.

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Tables and Figures:

Table 1: Composition of different Media Used in the Experiment

Ingredients	Medium-1	Medium-2	Medium-3	Medium-4
BSA (FAF)	30 mg	30 mg	30 mg	30 mg
FBS	20 ml	10 ml	20 ml	20 ml
LIF	400 ng	400 ng	400 ng	400 ng
ITS	0.1 mg	0.1 mg	0.1 mg	0.1 mg
IGF-I	1000 ng	1000 ng	1000 ng	1000 ng
bFGF	40 ng	40 ng	40 ng	40 ng
EAA	1 ml	1 ml	-	1 ml

Table 1 – Cond.,				
NEAA	0.5 ml	0.5 ml	1 ml	0.5 ml
Glucose	-	-	-	10 mg
CR11aa	Ad 100 ml	Ad 100 ml	Ad 100 ml	Ad 100 ml
NB: BSA (FAF): Bovine serum albumin fatty acid free; FBS: Fetal calf serum; LIF: Leukemia inhibitory factor; ITS: Insulin transferase selenium; IGF-I: Insulin like growth factor-I; bFGF: Bovine fibroblast growth factor; EAA: Essential amino acids; NEAA: Non-essential amino acids; Ad: Add up to				

Table 2: Primers used for PCR

Primer	Size	Sequence	Annealing temperature
β-actin	369bp	For-5'-GCGGGAAATCGTTCGTGACATCAA-3' Rev-5'5-TTGATCTTCATTGTGCTGGGTGCC-3'	56°C
Oct-4	398bp	For-5'-AAGCAGYGACTACTCCCAACGTGA-3' Rev-5'-TGAACTTCACCTTCCCTCCAACCA-3'	56°C
Nanog	211bp	For-5'-GGGAAGGGTAATGAGTCCAA-3' Rev-5'-AGCCTCCCTATCCCAGAAAA-3'	55°C

Table 3: Performances of Embryos Developed to Cell Clones in different Media And Culture Conditions

Media	Embryos developed to cell clones					
	Gelatin coating (n=32)		Matrigel coating (n=32)		Polylysine coating (n=32)	
	No.	%	No.	%	No.	%
Medium-1	13	40.625	15	46.875	0	0.0
Medium-2	11	34.375	10	31.25	0	0.0
Medium-3	15	46.875	15	46.875	0	0.0
Medium-4	11	34.375	12	37.5	0	0.0

"t" test reveals that there is no significant difference between media and culture condition.
NB: Polylysine was not considered for statistical analysis

Figures

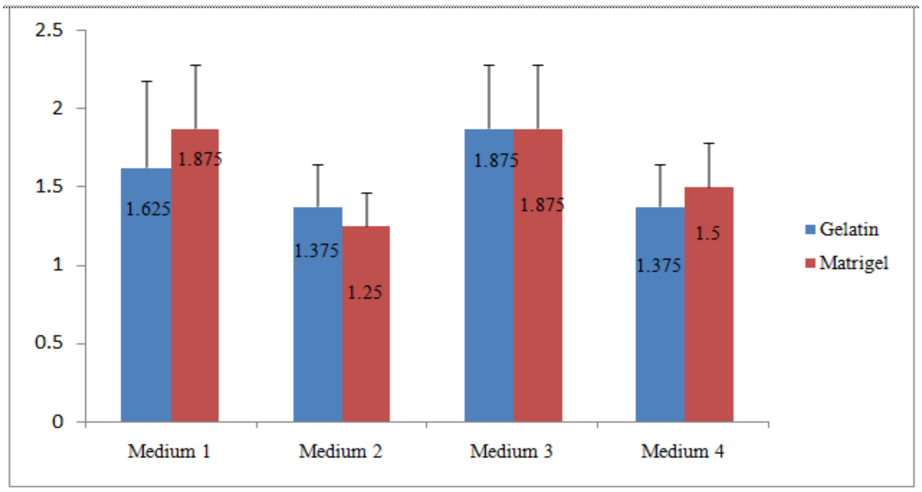

Figure 1: Mean (±SE) Numbers of Embryos Developed to Cell Clones in each well (out of 4 embryos) in different Media

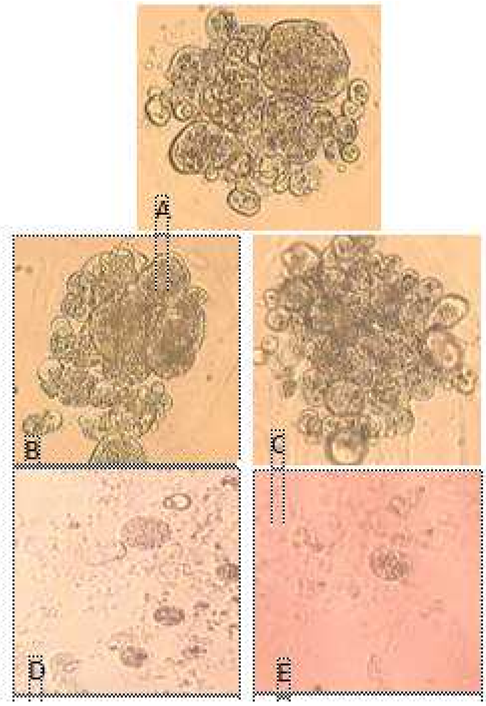

Figure 2: Cell Clone on Matrigel Coated Plate Derived from IVF Embryos

(A) Day 0 of Primary Culture (B) Day 2 of Primary Culture (C) Day 6 of Primary Culture (D) Day 6 of after First Passage (E) Day 6 of after Second Passage [10x10]

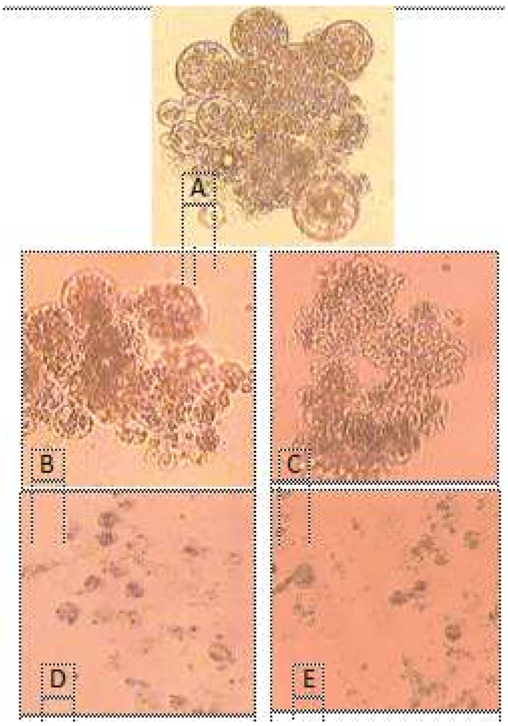

Figure 3: Cell Clone on Gelatin Coated Plate Derived from IVF Embryos

(A) Day 0 of Primary Culture (B) Day 2 of Primary Culture (C) Day 6 of primary culture (D) Day 6 after First Passage (E) Day 6 after Second Passage [10x110]

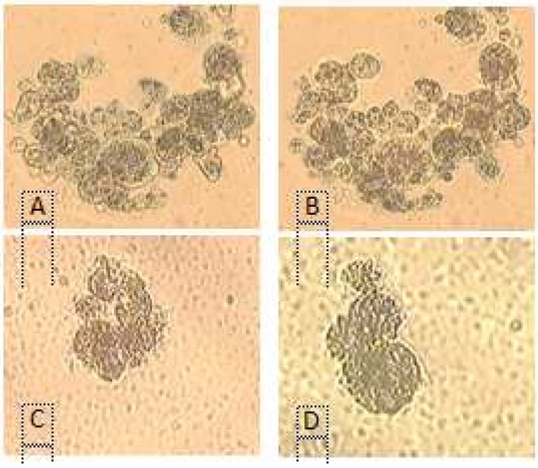

Figure 4: Blastomere Cells on Polylysine Coated Plate Derived from IVF Embryos Showing Gradual Detoriation

(A) Day 0 of Culture (B) Day 2 of Culture (C) Day 3 of Culture detoriated single Blastomere (D) Day 5 of Culture Detoriated Single Blastomere [10x10]

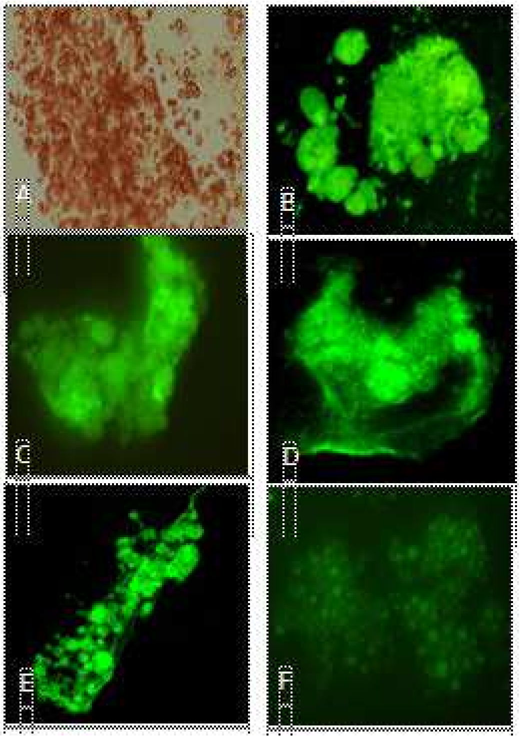

Figure 5: Characterization of Cell Clones by Stem Cell Markers

(A)Alkaline Phosphatase Positivity (B) SSEA-1 (C) SSEA-3 (D) TRA-1-81(80) (E) Oct-4 (F) Nanog [20x10]

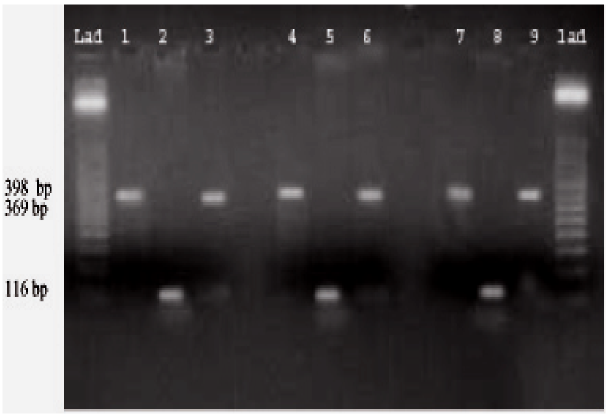

Figure 6: Visualization of PCR Product After Amplification for Oct-4, Nanog and β-actin

Lane Lad: 50bp ladder
Lane 1, 4, 7: Oct-4 of P0, P1, P2
Lane 2, 5, 8: Nanog of P0, P1, P2
Lane 3, 6, 9: β-actin of P0, P1, P2

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